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(54) Title: QUANTITATIVE VIRAL ASSAY (57) Abstract <p>A quantitative viral assay comprises capturing virus particles, releasing viral RNA or DNA and, where necessary, reverse transcribing viral RNA to complementary DNA (cDNA), amplifying the viral DNA or cDNA by a nested multi-stage PCR reaction, capturing the PCR product using an immobilised binding partner specific to a binding agent introduced in the PCR product and quantifying the captured PCR product. The assay is particularly useful in the determination of viral load and in the determination of the relative proportions of different viral forms, for example as produced by a point mutation. The assay may be used to follow the development of drug resistance.</p>		

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QUANTITATIVE VIRAL ASSAY

The present invention relates to a new viral assay which may be used either to measure the amount of virus in a serum sample or to determine the relative proportions of virus in
5 closely related viral forms, such as wild-type and a mutant caused by a point mutation at a single base in viral DNA or RNA.

Existing methods for estimating the amount of virus present in a serum sample rely on serial dilution in tissue
10 culture or polymerase chain reaction (PCR) systems. However known systems are labour intensive and time consuming. This renders them unsuitable for the investigation of the large number of specimens which are generated in clinic trials and in routine testing of patients subject to or suffering from viral
15 infection. Similarly, existing methods for the quantification of point mutations are inconvenient and cumbersome in that they involve the sequencing of whole DNA fragments, which is inconvenient and impractical on a large number of samples.

A new quantitative assay has now been devised which can
20 be used for both these applications, which possesses significant advantages in terms of convenience and the time involved. This may render the assay suitable for use in clinical trials, for example in determining whether a virus is developing resistance to a drug by following both the
25 proportion of mutated virus compared to unmutated (wild type) virus and the quantity of virus in the serum removed from a patient.

Accordingly the present invention provides a quantitative viral assay which comprises:

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- (a) capturing virus particles from serum and removing the residue of the serum;
- (b) releasing viral RNA or DNA from the virus particles;
- (c) where necessary, reverse transcribing viral RNA to complementary DNA (cDNA) using a transcription primer having a sequence complementary to a portion of the RNA sequence;
- (d) amplifying the viral DNA or cDNA by a nested multi-stage polymerase chain reaction (PCR) using in a first stage a first pair of PCR primers and in the final stage a second pair of PCR primers complementary to portions of the DNA sequence which lie within a region defined by the portions of the DNA sequence complementary to the first pair of PCR primers; one of the second pair of PCR primers being tagged with a specific binding agent capable of binding to a specific binding partner; and where reverse transcription is used to obtain complementary cDNA, the first pair of PCR primers having sequences complementary to portions of the cDNA which define a region such that the portion of the cDNA sequence complementary to the sequence of the transcription primer lies outside that region; and
- (e) capturing the PCR product tagged with a specific binding agent using an immobilised specific binding partner and quantifying the captured PCR product.

Preferably the assay is used to quantify an RNA virus, such as human immunodeficiency virus (HIV) and in particular HIV-1, in which case it is necessary to perform the reverse transcription step (c) to obtain complementary DNA before the

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PCR step (d).

The virus particles may be captured from the serum by known methods for example using affinity capture by a surface e.g. the surface of microtitre well, coated with binding agent, such as antibody, specific to the virus particles.

Preferably however the virus particles are captured using a suspension of fine particles coated with binding agent specific for the virus particles. These possess the advantages of a high surface area and short path length from the virus particles in the serum and they are readily separable from the residues of the serum, for example by centrifugation.

In a first embodiment of the invention the assay is used to quantify the amount of virus present in a sample. In this embodiment each stage of the PCR is performed over a predetermined number of cycles and therefore there is a known relationship between the final amount of amplified DNA and the amount of DNA prior to the PCR. The final stage of the PCR is performed in the presence of at least one labelled deoxynucleotide triphosphate (dNTP) or using a labelled primer so that the amplified DNA is labelled, the amount of labelling being proportional to the amount of DNA generated in the PCR. The reaction mixture is then contacted with a solid support bearing a binding partner specific to the specific binding agent tagged to the amplified DNA (which originates from the tagged primer in the second stage of the PCR), such that the amplified tagged DNA is bound by the specific binding partner to the substrate. Where the final stage of the PCR is performed using a labelled primer, the unlabelled primer is tagged with the specific binding agent, rather than

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the labelled primer so as to prevent binding to the substrate
f unamplified labelled primer which may lead to a false
result. The amplified DNA can then be separated from the
remainder of the unincorporated labelled dNTP or primer which
5 is removed for instance by washing. The amount of label in the
amplified DNA is then assayed and from this the amount of viral
or complementary DNA present before the PCR and hence the
amount of virus present in the serum is determined by
comparison with standard curves constructed using known amounts
10 of viral RNA or DNA or cDNA or virus.

The amplified DNA bound to the solid support is initially
in double stranded form. Optionally, before assaying the
amount of label incorporated in the amplified DNA, it is
denatured, the strands separated and the amount of label is
15 measured which has been incorporated in one of the single
strands, either the strand which is not bound to the support or
more preferably the single strand which is bound to the support
after denaturation.

Thus in one embodiment the invention provides a
20 quantitative viral assay which comprises

- a1) capturing RNA virus particles from serum using a
suspension of fine particles coated with a binding
agent specific to the virus particles;
- a2) separating the captured virus particles from the
25 serum;
- b) releasing viral RNA from the virus particles;
- c) reverse transcribing viral RNA to cDNA using a
transcription primer having a sequence
complementary to a portion of the RNA sequence;

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- d) amplifying the cDNA in a nested multi-stage polymerase chain reaction (PCR) using in a first stage a first pair of PCR primers having sequences complementary to portions of the cDNA sequence which define a region of the cDNA, such that the portion of the cDNA sequence complementary to the sequence of the transcription primer is outside that region; and in a second stage a second pair of PCR primers complementary to portions of the cDNA sequence which lie within the region defined by the portions of the cDNA sequence complementary to the first pair of PCR primers; one of the second pair of PCR primers being tagged with a specific binding agent capable of binding to a specific binding partner; the stages of the PCR being performed over a predetermined number of cycles such that amount of amplified DNA is proportional to the amount of cDNA prior to the PCR and the final stage being performed in the presence of a labelled deoxynucleotide triphosphate (dNTP) or using a labelled PCR primer;
- e) contacting the amplified DNA with a solid support bearing a binding partner specific to the binding agent tagged to the amplified DNA, permitting the amplified DNA to bind to the substrate, and removing unincorporated labelled dNTP or unincorporated labelled primer; and
- f) determining the amount of label in the amplified DNA and obtaining the amount of virus particles or

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viral RNA or cDNA in the serum by comparison with a standard curve.

Such a method may also be adapted to the assay of DNA virus.

- 5 In a second embodiment, the assay is used to quantify the relative proportions of two or more closely related forms of a virus differing by mutation at at least one known position in the viral RNA or DNA, such as a wild-type and a mutant. Such a mixture may result for example from a point mutation. The
- 10 mixture is subjected to viral capture, if necessary reverse transcription, and a multi-stage nested PCR; in the PCR it is not necessary to use labelled dNTP or primer. The reaction mixture is then contacted with a solid support bearing a binding partner specific to the specific binding agent tagged
- 15 to the amplified DNA, which originates in one of the primers in the second stage of the PCR, and then the amplified DNA is bound to the substrate. Any unincorporated dNTP is then removed and the DNA bound to the substrate is denatured to separate the strands. The assay then involves the addition of
- 20 a labelled nucleotide to an oligomeric probe annealed to a target sequence within the bound single stranded DNA fragment, the target sequence, having its 5' end at the base immediately adjacent to, i.e. one base short of, the target site. The 3' end of the probe is thus complementary to the base of the
- 25 target sequence immediately adjacent the target site and the base added to the probe therefore corresponds to the base at the target site. Assay of the type of nucleotide which is added to the probe identifies the nucleotide in the DNA fragment at the mutation site and quantification of the

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relative proportions of different nucleotide allows the proportion of the viral forms in the original mixture to be determined.

The uses of this process are not limited to assaying point mutations, and it will become apparent that it may be applied in other ways to the assay of DNA fragments.

Thus, in a second embodiment the invention provides a method of assaying the relative proportions in a mixture of forms of an RNA virus differing by mutation at at least one base in the viral RNA or DNA, which comprises:

- a1) capturing RNA virus particles from serum using a suspension of fine particles coated with a binding agent specific to the virus particles;
- a2) separating the captured virus particles from the serum;
- b) releasing viral RNA from the virus particles;
- c) reverse transcribing viral RNA to cDNA using a transcription primer having a sequence complementary to a portion of the RNA sequence;
- d) amplifying the cDNA in a nested multi-stage polymerase chain reaction (PCR) using in a first stage a first pair of PCR primers having sequences complementary to portions of the cDNA sequence which define a region of the cDNA such that the portion of the cDNA sequence complementary to the sequence of the transcription primer is outside that region; and in a second stage, a second pair of PCR primers complementary to portions of the cDNA sequence which lie within the region defined

by the portions of the cDNA sequence complementary to the first pair of PCR primers; one of the second pair of PCR primers being tagged with a specific binding agent capable of binding to a specific binding partner;

- 5 e) contacting the amplified DNA with a solid support bearing a binding partner specific to the binding agent tagged to the amplified DNA, permitting the amplified DNA to bind to the substrate, removing unincorporated dNTP and denaturing the amplified
- 10 DNA to separate the DNA strands;
- f) annealing to one of the strands of the amplified DNA a single stranded DNA oligomer probe having a sequence complementary to a target sequence within
- 15 the DNA fragment, the target sequence having its 5' end at the base immediately adjacent to the base to be analysed to form a probe/target strand duplex,
- g1) incubating a first portion of the probe/target
- 20 strand duplex strand with a DNA polymerase enzyme and labelled deoxynucleotide triphosphate (dNTP) or dideoxynucleotide triphosphate (ddNTP) corresponding to the base to be analysed in a first of the viral forms in the mixture;
- 25 g2) incubating a second portion of the probe/target strand duplex strand with a DNA polymerase enzyme and labelled deoxynucleotide triphosphate (dNTP) or dideoxynucleotide triphosphate (ddNTP) corresponding to the base to be analysed in a

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second of the viral forms in the mixture;

h) removing the unincorporated labelled dNTP or ddNTP;
and

5 i) determining the relative amounts of label bound to
the probe in each portion of the probe/target
strand duplex or in each portion of the probe after
denaturation and separation from the target strand.

The two steps g1) and g2) may be repeated a third
and optionally a fourth time using different labelled dNTP's or
10 ddNTP's. In addition to detect different variations in viral
forms at other potential mutation sites the procedure may be
repeated using other appropriate PCR primers and probes.

Figure 1 shows the results of experiments described in
detail in Example 1 to correlate the amount of ^{125}I label in PCR
15 product (expressed as test:negative ratio) against (a) the
dilution of HIV-1 cDNA in water (•) and (b) the dilution of
viral-containing serum in normal human serum (○ and □).

Figure 2 illustrates the principle used in the embodiment
of the invention relating to the assay to determine the
20 relative proportions of different viral forms in a mixture.

Figure 3 shows the point mutations in HIV-1 reverse
transcriptase (RT) and the locations of the relative probes and
primers used in the experiments described in detail in Example
2.

25 Figure 4 shows a calibration curve from the proportion of
Thr to Phe mutation at codon 215 in the RT gene of HIV-1 in
plasmid DNA as described in Example 2.

Figure 5 illustrates the use of the present invention in
determining the development over time of drug resistance

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associated with a mutation. The Figure shows the proportion of total HIV-1 viral genome carrying a zidovudine associated resistant mutation at codon 70 of the reverse transcriptase (RT) gene of HIV-1, in the serum (serum RNA ●) and in peripheral blood leucocytes (proviral DNA ■) in a patient versus the length of time on zidovudine treatment.

As already mentioned, the viral particles in the serum may be captured using known techniques. Preferably however the viral particles are captured using a suspension of fine particles, such as polystyrene particles, coated with binding agent, such as antibody specific for the virus particles. Preferably the particles are coated with antibody specific to envelope proteins of the virus particles, such as antibodies to the envelope protein gp120 of HIV-1.

Where such a method is used preferably the capture is performed using latex beads having a diameter from 0.1 to 20 μ m, preferably 0.5 to 1 μ m and most preferably 0.7 to 0.8 μ m. The beads may for example be formed from polystyrene or styrene divinyl benzene, optionally modified by carboxylation. In addition the beads may comprise magnetic material to aid in separation.

After the capture of the virus particles the residue of the serum is removed, for example by centrifugation of the suspended particles and removal of supernatant. Generally it will be necessary to wash the virus particles in physiologically acceptable buffer, e.g. phosphate buffered saline, to ensure all residual serum is removed. For example fine particles coated with the virus may be resuspended in buffer, and re-centrifuged.

The viral RNA or DNA is then released from the virus

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particles. This may be achieved in conventional manner using for example a detergent such as Triton X-100. Where the virus is a DNA virus the next step in the assay is the PCR amplification. However, where the virus is an RNA virus, the RNA is first reverse transcribed using a reverse transcriptase. Since RNA is easily degraded once released, it is preferred to have the reverse transcriptase enzyme and dNTP substrates already present when the RNA is released, or to add them together with the agent releasing the RNA. It is also preferable to employ together with the reverse transcriptase, one or more agents to prevent the degradation of the RNA, such as an inhibitor of RNAase. In this way the RNA is not exposed to an environment which will be likely to cause degradation of it before reverse transcription occurs.

Where reverse transcription is performed in order to produce cDNA, a primer is used for the reverse transcription which preferably has a sequence complementary to a portion of the cDNA outside a region of the cDNA defined by portions of the cDNA complementary to the first pair of PCR primers. Use of the reverse transcription primer outside the region defined by the PCR primers produces a more specific and quantitative PCR product than when the transcription primer is the same as one of the PCR primers.

Preferably the transcription primer is an oligomer of from 15 to 30 nucleic acids, and the reverse transcription is performed at a temperature from 20 to 70°C more preferably about 37°C, and for 30 minutes to 18 hours more preferably about 90 minutes.

Alternatively, a mixture of oligomers, such as hexamers,

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of random sequence may be used as primer for reverse transcription.

The viral DNA or cDNA thus obtained is amplified using a "multiple", for example "double", PCR. Typically the amount of starting DNA or cDNA will be from one to one million, for example about 1,000, molecules. The use of the multi-stage PCR procedure provides a specific amplification procedure from this very small amount of material. It will be appreciated that in order to perform the PCR it is necessary to know at least partially the sequencing of flanking regions of the DNA being amplified in order to design the primers. Typically in each strand of DNA, the portions which are complementary to the primers for each stage of the PCR are separated by a sequence from 20 to 1,000, for example 80 to 1,000, base pairs in length.

The PCR may be performed on the viral DNA or cDNA using known methods. Typically the PCR is performed using DNA priming oligomers of from 15 to 30, more preferably 20 to 25, nucleic acids. The reaction is typically cycled between three temperatures of (a) from 92 to 96°C, to denature the double stranded DNA, (b) from 37 to 72°C to anneal the primers to the DNA strands and (c) from 68 to 74°C to extend the DNA primers. However only two temperatures are required if the second and third steps are conducted at the same temperature. The reaction is preferably performed using a high temperature resistant DNA polymerase such as that of Thermus aquaticus, to avoid the need to add additional DNA polymerase during the reaction. It may, for instance, be performed in a reaction volume from 10 to 200 μ l, preferably 25 to 100 μ l.

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Quantification of amount of virus

Where the present invention is used to quantify the amount of virus in serum, all stages of the PCR are performed for a predetermined number of cycles such that the amount of PCR product bears a known relationship to the amount of starting DNA/RNA; i.e. the PCR is not allowed to reach a stage at which the amount of product becomes saturated and no longer increasing at which point the amount of product is no longer proportional to the amount of starting DNA.

Where the amount of starting viral or cDNA is small, e.g. up to about 1,000 molecules, the initial stage or stages of the PCR is preferably performed for 25 to 40, more preferably about 30 to 35, cycles.

The final stage of the PCR may be performed in the presence of at least one labelled dNTP. All of that dNTP may be labelled or a mixture of the labelled dNTP with the corresponding unlabelled dNTP may be used provided that the proportion of these are known. Any type of conventional labelling may be used, such as radiolabelling, for example with ^{32}P , ^{35}S or ^{125}I or fluorescence labelling for example with fluorescein, rhodamine or an enhanced fluorescence labelling system employing for instance Europium. It is especially preferred to use ^{125}I labelling, e.g. using ^{125}I -dCTP, as this is found to have a dynamic range especially suited to the assay.

Alternatively rather than using labelled dNTP, the PCR may be performed using a labelled primer. Such a primer may be labelled using conventional labelling techniques. Specific examples of labels which may be used include digoxigenin and

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FITC labels and sulphonated bases in the primer.

Preferably the final stage of the PCR is performed over 3 to 20, more preferably 5 to 15 cycles. In an especially preferred embodiment the final stage of the PCR is performed over a fixed number of cycles and interrupted at equal intervals, e.g. by removing a portion of the product after a number of cycles, so as to optimise the number of cycles for comparison against a standard curve. For example, the PCR may be performed over 15 cycles and interrupted for sample removal after 5 and 10 cycles, so as to obtain a result which is in the most sensitive, preferably linear, region of a standard curve.

After the PCR, the amplified DNA is separated from unwanted components by use of a specific binding interaction. For this purpose one of the PCR primers used in the final stage of the PCR is tagged with a specific binding agent, such as biotin, preferably at the 5'-terminus. After the PCR, the amplified DNA is contacted with a solid support which bears a specific binding partner (such as streptavidin) for the specific binding agent which causes specific binding of the amplified DNA via the tagged primer to the solid phase. The unwanted components of the amplifying reaction mixture and in particular unincorporated labelled dNTP may then be washed away.

Preferably, only one of the PCR primers is tagged with a specific binding agent so that only one strand of the amplified DNA will be directly attached to the solid phase.

Suitable binding agent/partner combinations include: antigen/antibody pairs; biotin/streptavidin and biotin/avidin. It will be apparent that of these combinations, either member

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may be attached to the DNA and the other member to the support. A particularly favoured combination is the use of biotin attached to the 5' end of the PCR primer and streptavidin or avidin on the support.

5 Suitable solid phases include microwells, polystyrene spheres, (e.g. 6mm polystyrene spheres), magnetic latex beads (eg. Dynabeads), non-magnetic latex beads, nylon and nitrocellulose membranes.

 The incorporation of the label into the PCR is then
10 measured, e.g. by measuring the radioactivity or fluorimetry. When the PCR has been performed using a labelled primer such as one labelled with digoxigenin, FITC or sulphonate labelling, detection may be by an immunoassay using monoclonal antibodies specific to the label.

15 The measurement of label may be made on the PCR product attached to the solid phase. Alternatively the strands of the DNA product may be separated by denaturing the strand not attached to the solid support by the specific binding interaction isolated and the label incorporated into that
20 strand measured.

 A standard curve may be constructed to determine the amount of label incorporated into the PCR product using known amounts of virus particle under the same conditions as those used to assay serum samples. By comparison with such a curve
25 the amount of virus particle present in serum can be quantified. Similarly by comparison with curves constructed using known amounts of viral RNA, DNA or cDNA the amount of those present in a sample can be determined.

Quantification of Relative Proportions of Viral Forms in a

Mixture

When the method of the present invention is used to quantify such proportions, it is necessary that the sequence (hereafter the target sequence) immediately adjacent the base to the analysed (which differs in the different viral forms and is hereafter referred to as the target site) is also known to allow design of a probe. It is also necessary that sequences of regions flanking the target sequence be known to allow the design of PCR primers.

Both stages of the PCR used in this embodiment are performed using unlabelled dNTP's, though in the final stage a primer tagged with a specific binding agent is employed. This produces double stranded PCR product tagged on one strand by a specific binding agent, preferably at the 5' end, (see Figure 2A). Preferably each stage of the PCR is performed over 25 to 45, more preferably 30 to 40 cycles.

After the PCR, the amplified DNA is separated from unwanted components by use of a specific binding interaction (Figure 2B). Prior to this the amplified DNA is split into more than one portion, preferably into four separate portions, each of which is intended for incubation separately with a different labelled dNTP or ddNTP.

To provide the specific binding interaction, one of the PCR primers used in the final stage of the PCR is tagged with a specific binding agent, such as biotin, preferably at the 5'-terminus. After the PCR, the amplified DNA is contacted with a solid support which bears a specific binding partner (such as streptavidin) for the specific binding agent which causes specific binding of the amplified DNA via the tagged primer to

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the solid phase. The unwanted components of the amplifying reaction mixture and in particular unincorporated dNTP may then be washed away.

Only one of the PCR primers is tagged with a specific binding agent so that only one strand of the amplified DNA will be directly attached to the solid phase. When the DNA is denatured to separate the strands the other strand may then be removed by washing. If both primers were tagged, the denatured amplified DNA could reanneal when denaturing conditions are removed and this would interfere with subsequent detection of the target site.

Suitable binding agent/partner combinations include: antigen/antibody pairs; biotin/streptavidin and biotin/avidin. It will be apparent that of these combinations, either member may be attached to the DNA and the other member to the support. A particularly favoured combination is the use of biotin attached to the 5' end of the PCR primer and streptavidin or avidin on the support.

Suitable solid phases include microwells, polystyrene spheres, (e.g. 6mm polystyrene spheres), magnetic latex beads (eg. Dynabeads), non-magnetic latex beads, nylon and nitrocellulose membranes.

The DNA attached to the solid support is then denatured to separate the strands (Figure 2C). This may for example be achieved by subjecting the PCR product to alkaline conditions for instance at a pH of at least 10 or higher, e.g. with sodium or potassium hydroxide or by heating the amplified product, for instance to a temperature from 94 to 100°C, preferably about 95°C. The conditions will be selected so as to avoid

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denaturing the specific binding agent and partner and disrupting the specific binding thereof.

After the denaturation step, the single stranded amplified product, attached to the solid support, containing
5 the target sequence is annealed to the single stranded oligomeric DNA probe to form a probe/target strand duplex (Figure 2D).

The probe is typically 20 to 30 bases in length.

The probe's sequence corresponds to the target sequence
10 immediately adjacent to the target site in the 5' direction.
-- In the case of analysis of a point mutation the target site is the point of mutation; in other cases it is simply the base to be analysed.

In certain cases, it may be possible to select the PCR
15 primers so that the target site is one base downstream of the 3' terminus of one of the primers used in the PCR, in which case that primer may also be used as the probe.

The annealing of the probe to the target sequence may be performed at elevated temperature typically using an excess of
20 probe. Typically the temperature will be from 50 to 60°C and this temperature is maintained for half a minute to 5 minutes, and the solution is then allowed to cool slowly.

After annealing, the probe/target strand duplex is incubated with a single labelled dNTP or preferably ddNTP and a
25 DNA polymerase enzyme such as Klenow DNA polymerase or T7 DNA polymerase, for instance Sequenase (Figure 2E). If the labelled dNTP or ddNTP is complementary to the base of the target site labelled dNTP or ddNTP will be added to the probe, otherwise the labelled dNTP or ddNTP will not normally be

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incorporated into the probe. This therefore allows the target site base to be identified.

If, as is preferred, a labelled ddNTP is used rather than a labelled dNTP this will prevent further labelled nucleotides being added to the primer after a first labelled base is added. Thus if the sequence of the target strand which is downstream of the 3' end of the probe contains repeated bases corresponding to the labelled nucleotides then with dNTP multiple labelled bases will be added but with ddNTP only a single labelled base is incorporated.

Any conventional label may be used in the labelled dNTP or ddNTP, such as a radioactive label, for example ^{32}P or ^{35}S , or a fluorescent label, such as fluorescein or rhodamine.

The separate samples of probe/target strand duplex are preferably treated with different labelled dNTP's or ddNTP's bearing the same label to allow evaluation of the relative proportions of the sample which incorporate the different nucleotides. In some cases different labels may be usable with the different dNTP's, for example different colourimetric labels may be used.

After incubation with labelled dNTP or ddNTP the unincorporated labelled dNTP or ddNTP is separated from the probe/target duplex. As the probe/target duplex is attached to a solid phase, this may be achieved by washing.

Finally the extent of incorporation of the label into the probe is measured, e.g. by measuring the radioactivity or by fluorimetry. This detection or measurement may be made on the probe/target strand duplex or, preferably on the probe alone which may be separated from the target strand by denaturation

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(Figure 2F), e.g. in alkaline conditions. Detection may for example be performed using a scintillation counter on a sample in a microtitre well.

After denaturation of the probe/target duplex, the target
5 strand may be recycled by washing in buffer and then annealed to a second probe for analysis of the base at a second target site using the procedure described above. The target strand may be stored in a buffer for up to several days before performing such a further assay. The procedure may be further repeated
10 for assay at further target sites.

Using the method of the present invention it is possible to assay for a point mutation, and determine the relative proportions of viral forms in a mixture, e.g. the extent of mutation in a mutated/unmutated mixed population by comparison
15 of the extent of incorporation of different labelled dNTP's or ddNTP's. Where, in one of the viral forms, the base at the target site is repeated in one or more immediately succeeding bases this leads to the incorporation into the probe of more than one base and therefore to an increased incorporation of
20 label. This increased incorporation of label will be taken into account in determining the relative proportions of the different viral forms in the mixture.

It will be appreciated that the quantification of amount of virus and of relative proportions of viral forms in a
25 mixture may be used in combination to give a more detail analysis of a serum sample. Thus before the PCR step, the sample may be divided into two portions one of which is assayed for the amount of virus and the other for the relative proportions of viral forms.

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The invention will be further illustrated by reference to the following Examples:

Example 1

5 Six uninfected patients and 29 infected patients at various stages of HIV-1 disease defined by the Centre for Disease Control (CDC) classification including 6 CDC group II; 9 CDC group III and 14 CDC group IV patients were sampled as a cross-sectional study.

10 Blood samples were collected from patients, allowed to clot for no more than 1.5 hours at room temperature, separated and the sera stored at -70 °C for up to 4 years prior to this study.

Preparation of Latex Pellets

15

For the assay of serum/plasma HIV-1 RNA, latex microparticles were coated with anti-HIV-1 antibody. A ten percent latex solution (Sigma Limited, one volume) was washed three times in 0.1 M Tris-HCl buffer (pH 7.6, 50 volumes) and
20 pelleted by centrifugation (10,000 g for ten minutes). The latex pellet was then resuspended in Tris-HCl buffer, (50 volumes) containing one monoclonal and four polyclonal antibodies (80 µg/ml each) raised against recombinant surface envelope glycoprotein of HIV-1 (anti-gp-160/120) and incubated
25 with gentle shaking at room temperature (24 hours). The coated latex was pelleted (7,000 g for six minutes) washed three times as above and was finally resuspended in 0.1 M Tris-HCl buffer (pH 7.6, 35 volumes) containing Sodium Azide (0.1%).

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Viral Capture and Reverse Transcription

To capture virus and produce HIV-1 cDNA sera first were spun to remove any cellular fragments (15,000 g for five minutes). Serum (100 μ l) was then removed and incubated with gentle shaking for one hour at room temperature together with the anti-HIV-1 coated latex solution (20 μ l). The latex/virus complex was then pelleted (7,000 g for four minutes), washed in phosphate buffered saline (PBS, 100 μ l), and pelleted (7,000 g for four minutes). The washed pellet was resuspended in a solution (20 μ l) containing Triton-X-100 (0.1%), reverse transcriptase primer CP1 (5'GGAGGGGTATTGACAA3') (0.1 μ mol/l), Hepes-HCl (pH 6.9, 5 mmol/l), EDTA (pH 8, 0.1 mmol/l), Tris-HCl (pH 7.5, 50 mmol/l), KCl (75 mmol/l), MgCl₂ (3 mmol/l), DTT (10 mmol/l), each dNTP (0.5 mmol/l each), RNase inhibitor (22 units) (Pharmacia Ltd), and recombinant Moloney Murine Leukemia Virus reverse transcriptase (Pharmacia Ltd) (15 units). The mixture was incubated for 90 minutes at 37°C.

20 Amplification and Quantification of cDNA

cDNA was amplified and quantified by two rounds of PCR, using nested primers in the pol gene. Ten microlitres of patient derived cDNA were amplified in the first round of PCR in 50 μ l of reaction mix using the outer primers:

25 MH5 5'GCAGGGGCAAGGCCAATGGACAT3'
 MH6 5'CTCCCACTCAGGAATCCAGGTGGC3'

Two microlitres of the first round products were amplified by a second PCR using inner primers:

POL1 biotinylated (5'CAGGAAAATATGCAAGAATGAGG3')

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POL2 (5'CCCATGTTTCCTTTTGTATGGGT3').

Both reactions were performed in a total reaction mix (50 μ l) containing Tris-HCl (pH 8.3, 10 mmol/l), KCl (50 mmol/l), MgCl₂ (1.5 mmol/l), gelatin (0.01% w/v), Tag polymerase 5 ("Amplitaq"; Perkin Elmer Cetus, 1.25 units) and primers (0.1 μ mol/l each).

The first PCR was performed using a concentration of 200 μ M of each dNTP in the reaction mixture. Thermal cycling consisted of 1 cycle at 94°C for 4 minutes; 35 cycles at 94°C 10 for 1 minute, 60°C for 1 minute and 72°C for 1 minute then finally 1 cycle at 72°C for 7 minutes.

The second PCR was performed using dGTP (20 μ mol/l), dATP (20 μ mol/l), dTTP (20 μ mol/l), ¹²⁵I dCTP (NEN Dupont (10 nmol/l) and primers (BioPOL1 and POL2) (0.1 μ mol/l each). Thermal 15 cycling consisted of a total of 15 cycles at 94°C for one minute, 50°C for one minute and 72°C for one minute.

PCR product (10 μ l) was removed from the second PCR reaction after five, ten and fifteen cycles and separately added to streptavidin-coated *removawells* (Dynatech Ltd) 20 containing Tween-20 (0.05%) in PBS (90 μ l). After incubation at room temperature (30 minutes) the wells were washed thoroughly with Tris-HCl (pH 7.6, 0.01M) containing Tween -20 (0.05%), and the binding of ¹²⁵I labelled DNA measured by gamma counting.

25 Results

HIV-1 viral RNA was also measured by precipitation with polyethylene glycol (PEG) and cDNA quantified by end-point titration in a PCR as previously described (Semple M, Loveday C, Weller I and Tedder R (1991) Journal of Medical Virology,

35, 38-45).

The immune affinity assay was standardised by deriving test: negative ratios (T:N) for ^{125}I binding for all specimens. Pooled normal human serum (NHS) from blood donors, screened for 5 anti-HIV-1 antibodies was included in each assay as a negative serum control. Serum "M", from an HIV-1 infected patient, was aliquoted and included in each assay as a positive- serum control. In later assays, serum "M" and serum "K", both containing a known titre of detectable cell-free HIV-1 RNA, and 10 dilutions of these sera were used to construct regression lines. A dilution series of proviral HIV-1 CBL-1 DNA of known copy numbers/ml was included in all nested PCR reactions. T:N ratios for known numbers of copies of HIV-1 DNA at each concentration in the series were plotted to give standard 15 curves based on sampling after five, ten and fifteen cycles of second round PCR. The number of copies of HIV-1 cDNA generated by reverse transcription of experimental specimens was estimated by comparison with the standard DNA curves. A minimum copy number for HIV-1 RNA in the analyte was derived 20 from the cDNA copy per ml value assuming that one RNA genome generated a single cDNA copy.

To control against the possibility of amplifying proviral DNA which may absorb non-specifically to the coated latex, serum samples from 20 HIV-1 infected patients were incubated 25 with the latex solution (uncoated with antibodies) as described. The latex was pelleted, washed and resuspended in a PCR buffer containing Triton-X-100 (0.1%) and subjected to the above quantitative PCR protocol.

To estimate intra-assay reproducibility serum "M" was

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measured nine times in one assay and the values compared (mean, range and standard deviation). To estimate inter-assay variability, the same serum was measured in a sequence of 11 assays and the results compared (mean, range and standard
5 deviation).

Sera were tested for markers of virus infection by known methods. No proviral DNA was detected in any of the 20 serum samples assayed from HIV-1 infected patients. For the quantification of cDNA a linear dose response curve was found
10 when ^{125}I binding was related to DNA copies/ml; Figure 1a shows ^{125}I binding, expressed as a test:negative ratio versus dilutions of HIV-1 proviral DNA in water. Furthermore, ^{125}I binding was also linearly related to the titre of HIV-1 RNA in the test serum analysed; Figure 1b shows ^{125}I binding, expressed
15 as a test:negative ratio for dilutions of HIV-1 proviral DNA versus dilutions of sera "K" (\square) and "M" (O) in normal human serum. Good intra-assay reproducibility was demonstrated by the replicate results derived for serum "M". A mean titre (expressed as minimum copies of HIV-1 RNA per ml of serum:
20 c/ml) of 44,904 c/ml was obtained with a range of 1/3 of a \log_{10} (28,060 c/ml to 52,280 c/ml) and a standard deviation of 7,837 c/ml. In comparison the inter-assay variance was greater. The same positive serum sampled in a sequence of 11 assays gave a mean copy number of 31,153 c/ml with a range of 3/4 of a \log_{10}
25 (12,040 c/ml to 64,200 c/ml) and a standard deviation of 18,886 c/ml.

Plasma HIV-1 RNA concentrations in sera from the cross-sectional group of patients, measured by the immune-affinity assay and the PEG assay were compared. HIV-1 RNA was detected

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in all sera from infected individuals. The geometric mean titres of HIV-1 RNA in the samples measured by the PEG method were; 681 c/ml (CDC II, n=6), 774 c/ml (CDC III, n=9) and 8482 c/ml (CDC IV, n=14). The titres when measured by the novel immune-affinity quantitative PCR method were 241 c/ml, 374 c/ml and 25,523 c/ml, respectively. There was good correlation between the level of virus estimated by the immune affinity and the PEG methods (coefficient of correlation $r = 0.94$, $P > 0.001$). p24 antigen was detectable in only 14 of the 29 sera from infected individuals and of those 14 sera, only 3 came from patients in CDC groups II and III.

Example 2

Samples were analysed for point mutations in the reverse transcriptase (RT) gene of HIV-1. Four point mutations were assayed for their occurrence in codons 67, 70, 215 and 219 of the RT gene of HIV-1 and are shown in Figure 3.

The primers and probes used in the individual assays are listed below and their relative positions in the RT gene are shown in Figure 3.

- Primer SPP1 (21 mer) 5'A GGA CCT ACA CCT GTC AAC AT 3' (Sense)
 Primer MH6A (21 mer) 5'A CTC AGG AAT CCA GGT GGC TT 3' (anti-sense)
 Primer SPP2 (24 mer) 5'G TTG ACT CAG ATT GGT TGC ACT TT 3' (sense)
 Biotinylated for use with probe ARP1, ARP3, ARP4C
 * Primer SPP6 (23 mer) 5'TG GAG TTC ATA ACC CAT CCA AAG 3' (anti-sense)
 Biotinylated for use with probe ARP2B
 Probe ARP1 (20 mer) 5'TTT TCT CCA TTT AGT ACT GT 3' (anti-sense)
 For codon 67 wild-type adds C, mutant adds Tx7
 Probe ARP2B (20 mer) 5'A AAG AAA AAA GAC AGT ACT A 3' (sense)
 For codon 70 wild-type adds Ax2, mutant adds G
 Probe ARP3 (20 mer) 5'AAG TTC TTT CTG ATG TTT TT 3' (anti-sense)
 For codon 219 wild-type adds T, mutant adds Gx2
 Probe ARP4C (22 mer) 5'CTG ATG TTT TTT GTC TGG TGT G 3' (anti-sense)
 For codon 215 wild-type adds G, mutant adds Ax2 (Phe) or T (Tyr)

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Samples for Analysis

- DNA sequences were amplified from DNA extracted from Ficoll-paque separated PBMCs in an extraction buffer containing proteinase K, Tween 20 and NP40 (Higuchi 1989). Control
- 5 sequences were amplified from plasmid DNA (RTI/H) and RTMC supplied by Brendan Larder, Wellcome Research Laboratories, Beckenham, U.K.) or DNA extracted from a chronically infected MT-2 cell line (A012B and A012D-supplied by MRC AIDS Reagents Project).
- 10 RNA may be analysed by capture of viral particles on latex beads coated with antibodies specific to the virus, lysis of virus particles and reverse transcription to produce cDNA as described in Example 1.

15 PCR Amplification

- The sequences were amplified in a double PCR using nested primer sets. Thermal cycling for the first round PCR consisted of 1 cycle at 94°C for 4 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and 1 cycle at
- 20 72°C for 7 minutes. The second round ("nested") PCR consisted of 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and 1 cycle at 72°C for 7 minutes. Reactions were carried out in a reaction mixture (50µl) containing Tris/HCl (10mM, pH8.3), KCl (50mM), MgCl₂ (1.5mM), gelatin (0.01% w/v)
- 25 50 µl), Taq polymerase ("Amplitaq": Perkin-Elmer-Cetus; 1 unit), each dNTP (200µM each) and each primer (0.1µM each).

Microtiter Point Mutation Assay

PCR product (10µl aliquots) were diluted (to 25µl) with

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Tris/HCl buffer, (10 μ M, pH 7.6), containing Tween 20 (0.05% (TTB), and added to each of 4 Microtiter wells (Nunc, U-well) coated with Streptavidin (25 μ g/ml, 25 μ l/well), and incubated at room temperature (15 minutes) to capture the biotinylated PCR product.

The wells were washed (3 times) with TTB to remove the PCR reaction components and then NaOH (40 μ l, 0.15M) was added to each well for 5 minutes at room temperature to denature the captured PCR product. The wells were washed (4 times) with TTB to wash away the released second strand and anneal mix (25 μ l of 40mM Tris/HCl, pH7.6, containing 20mM MgCl₂, 50mM NaCl and 2.5 μ M oligonucleotide probe) were added to each well. The wells were heated in a waterbath (65°C), held at that temperature for 3 minutes and allowed to cool slowly to room temperature (30 minutes) to allow the probe to anneal. An aliquot (6 μ l) of labelling mix (40mM Tris/HCl, pH7.6, containing 16.6 μ M dithiothreitol, 0.016% BSA, 0.15 units Klenow DNA polymerase and 0.16 μ M of one ³⁵[S] labelled dNTP- 1200Ci/mmol) was added to each well, a different labelled dNTP being added to each of the 4 wells, and incubated at room temperature (2 minutes). The wells were then washed (5 times) with TTB, with a soak (1 minute) after the 4th wash, to remove unincorporated label. NaOH (40 μ l, 0.15M) was then added to each well to denature the probe together with any incorporated labelled nucleotide away from the target strand. The NaOH solution was removed and mixed with 5 ml scintillation cocktail (Optiphase Hisafe-3, LKB). The samples were counted for 1 minute in an LKB Minibeta.

As an alternative the buffer used in this procedure may

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additionally contain 0.1% sodium azide to inhibit bacterial contamination (such a buffer is designated TTA).

Results

- 5 The percentages of wild-type and mutant sequences were calculated after subtracting the non-specific background reading derived from the base or bases not found at the point being analysed (i.e. A and G at codon 67, C and T at codon 70, C at codon 215, A and C at codon 219). The radiolabel
- 10 incorporation for the mutant T addition at codon 67 is 7 bases hence the T signal in this case was divided by 7. Similarly the wild-type sequence at codon 70 incorporates 2 A residues and the Phe mutation at codon 215 incorporates 2 A residues and each of these signals was adjusted accordingly.
- 15 A standard curve for the Thr to Phe mutation at codon 215 was derived by mixing known DNA copy numbers of wild-type (RTI/H) and mutant (RTMC) plasmid. The plasmid samples were quantified by end-point dilution in a PCR according to the method of Simmonds et al. (Simmonds, P., Balfe, P., Peutherer,
- 20 J.F., Ludlam, C.A., Bishop, J.O. and Leigh-Brown, A.J. (1990): Journal of Virology, 64, 864-872) and the total number of DNA copies added to PCR reaction for analysis by the point mutation assay was 10^3 . The results are shown in Table I and Figure 4. Figure 4 shows a calibration curve for detection of the
- 25 mutation at codon 215, as the percentage of mutant plasmid detected versus percentage of mutant plasmid added.

The analysis of tissue culture derived control samples is shown in Table II. These samples were from isolates made at 2 months (A012B) and 26 months (A012D) after commencement of

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zidovudine therapy. Sample A012B showed approximately 100% wild-type sequence at all four codons, whereas A012D showed approximately 100% mutant sequence at codons 67, 215 and 219 and a 23.2% : 74.8% wild-type : mutant mixture at codon 70.

- 5 This method may be successfully used to detect incidences of mutation as low as 2% in a viral population.

TABLE 1

Assay standard curve showing deterioration of Thr to Phe mutation at codon 215 of the RT gene of HIV-1 in plasmid DNA

% wild-type plasmid (RTI/H) added	% mutant plasmid (RTMC) added	% mutant detected in assay
100	0	0
97	3	4.0
94	6	5.5
87.5	12.5	11.4
75	25	28.9
50	50	52.8
0	100	100

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TABLE 2

Codon	Sample	Signal (c.p.m.)				% wild-type	% mutant
		A	G	C	T		
a.a.67	A012B	36	133	5449	70	100	0
	A012D	31	134	97	570	1.1	98.9
a.a.70	A012B	2849	37	3	41	99.5	0.5
	A012D	627	1781	10	50	23.2	74.5
a.a.215	A012B	32	2350	12	18	99.4	0.4/0.2*
	A012D	5832	20	8	11	0.3	99.6/0.1*
a.a.219	A012B	41	18	17	314	100	0
	A012D	40	1006	13	34	0.8	99.2

* phe/tyr

Wild-type at a.a.67=C, mutant=T

Wild-type at a.a.70=A, mutant=G

Wild-type at a.a.215=G mutant=A(phe) or T(tyr)

Wild-type at a.a.219=T, mutant=G

A012B= isolate in MT2 cell culture from patient treated with zidovudine for 2 months (MRC AIDS reagent project)

A012D= isolate in MT2 cell culture from patient treated with zidovudine for 26 months (MRC AIDS reagent project)

Example 3

The reproducibility of the point mutation assay described in Example 2 for mutation of the codon 215 from Thr to Tyr was

investigated for four samples a, b, c and d in five repeated experiments. The results of these experiments shown in Tables 3 and 4 for the assay of cell-free viral RNA and pro-viral DNA respectively show little variation from run to run.

TABLE 3

Cell-free viral RNA assayed for mutation to Tyr at codon 215

Sample	Run 1	Run 2	Run 3	Run 4	Run 5	Mean %	S.D.
A	56	49	58	58	50	54.2	4.4
B	41	46	38	38	36	39.8	3.9
C	82	82	85	88	89	85.2	3.3
D	34	45	40	49	45	42.6	5.8

TABLE 4

Proviral DNA assayed for mutation to Tyr at codon 215

Sample	Run 1	Run 2	Run 3	Run 4	Run 5	Mean %	S.D.
W	8	25	12	11	17	14.6	6.6
X	9	10	11	6	5	8.2	2.6
Y	25	32	25	26	26	26.8	2.9
Z	45	39	41	31	38	38.8	5.1

Example 4

The method of Example 2 was used to assay for mutation at codon 215, using ARP4C as probe. After aspirating the samples were washed four times with TTB buffer and aspirated. The samples were then annealed to a second probe ARP1 to assay for mutation at codon 67 using the same procedure. The results of this repeated assay of a single probe are shown for two samples below:

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Sample 1

Probed with ARP4C for mutation at codon 215

G = 22860 cpm)	Wild Type = 99 %
A = 68 cpm)	Mutant to phe = 0%
T = 179 cpm)	Mutant to tyr = 1%
C = 27 cpm)	

Re-probed with ARP1 for mutation at codon 67

C = 19009 cpm)	Wild Type = 98%
T = 3500 cpm)	
A = 384 cpm)	Mutant = 2%
G = 79 cpm)	

Sample 2

Probed with ARP4C for mutation at codon 215

G = 10426 cpm)	Wild Type = 70%
A = 597 cpm)	Mutant (phe) = 2%
T = 4132 cpm)	Mutant (tyr) = 28%
C = 43 cpm)	

Re-probed with ARP1 for mutation at codon 67

C = 2060 cpm		Wild Type = 53%
T = 12825 cpm		
A = 51 cpm		Mutant = 47%
G = 63 cpm		

Example 5Point Mutation Assay at Codon 184 of the Reverse Transcriptase Gene

The identification of the three dimensional structure of the reverse transcriptase enzyme has led to the identification of various active sites of the enzymes and the identification of the positions of amino acids associated with drug resistance. The sequence of amino acids encoded by codons 183-186 (tyrosine, methionine, aspartic acid, aspartic acid) appears to be essential for enzymic activity and preparations in this sequence are associated with resistance in a number of non-nucleoside RT inhibitors.

Using a method analogous to that of Example 2, an assay has been developed for the mutation at codon 184 (methionine to valine) using as a probe:

5' TCCTACATACAAATCATCCA 3' (antisense)

wild-type adds T, (x1) mutant adds C (x1).

The results of a series of assays on mixtures of known proportions of wild type and mutant virus are shown in Table 5 below. The samples were mixtures of PCR product diluted to 10^3 copies/sample. The PCR product used in the sample was amplified from viral RNA, reverse transcribed to cDNA, from patients known to be 100% wild type or 100% valine mutants at codon 184. The samples were quantified by end point dilution as described by Simmonds et al (supra - page 29).

TABLE 5

Sample	Base	Reading	Background	Adjusted Reading	%
0% mutant	T	2627	38.5	2588.5	100
	C	22		<0	0
80% mutant	T	545	63	482	32
	C	1080		1017	68
50% mutant	T	1432	49.5	1382.5	58
	C	1053		1003.5	42
20% mutant	T	1685	42	1643	83
	C	375		333	17
10% mutant	T	2732	34.5	2697.5	93
	C	228		193.5	7
4% mutant	T	2534	43.5	2490.5	97
	C	120		76.7	3
2% mutant	T	3436	38	3398	98
	C	110		72	2
100% mutant	T	18	60	<0	0
	C	2911		2851	100

Background measured as mean level in samples assayed for

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incorporation of A and G.

Adjusted Reading is the measured level for the sample virus background.

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CLAIMS

1. A quantitative viral assay which comprises:
 - (a) capturing virus particles from serum and removing the residue of the serum;
 - (b) releasing viral RNA or DNA from the virus particles;
 - (c) where necessary, reverse transcribing viral RNA to complementary DNA (cDNA) using a transcription primer having a sequence complementary to a portion of the RNA sequence;
 - (d) amplifying the viral DNA or cDNA by a nested multi-stage polymerase chain reaction (PCR) using in a first stage a first pair of PCR primers and in the final stage a second pair of PCR primers complementary to portions of the DNA sequence which lie within a region defined by the portions of the DNA sequence complementary to the first pair of PCR primers; one of the second pair of PCR primers being tagged with a specific binding agent capable of binding to a specific binding partner; and where reverse transcription is used to obtain complementary cDNA, the first pair of PCR primers having sequences complementary to portions of the cDNA which define a region such that the portion of the cDNA sequence complementary to the sequence of the transcription primer lies outside that region; and
 - (e) capturing the PCR product tagged with a specific binding agent using an immobilised specific binding

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partner and quantifying the captured PCR product.

2. Assay according to claim 1 in which the virus is an RNA virus and in step (c) viral RNA is reverse transcribed providing cDNA.

3. Assay according to claim 2 in which the reverse transcription primer has a sequence complementary to a portion of the RNA sequence outside a region of the cDNA defined by portions of the cDNA complementary to the first pair of PCR primers.

4. Assay according to any one of the preceding claims in which virus particles are captured using a suspension of fine particles coated with binding agent specific for the virus particles.

5. Assay according to any one of the preceding claims which comprises:

in step (d) amplifying the cDNA or viral DNA in a nested multi-stage polymerase chain reaction (PCR) using in a first stage a first pair of PCR primers having sequences complementary to portions of the cDNA or viral DNA sequence which define a region of the cDNA or viral DNA, such that the portion of the cDNA or viral DNA sequence complementary to the sequence of the transcription primer is outside that region; and in a second stage a second pair of PCR primers complementary to portions of the cDNA or viral DNA sequence which lie within the region defined by the portions of the cDNA or viral DNA sequence complementary to the first pair of PCR primers; one of the second pair of PCR primers being tagged with a specific binding agent capable of binding to a specific binding partner; the stages of the PCR being performed over a

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predetermined number of cycles such that amount of amplified DNA is proportional to the amount of cDNA or viral DNA prior to the PCR and the final stage being performed in the presence of a labelled deoxynucleotide triphosphate (dNTP) or using a labelled PCR primer;

in step (e) contacting the amplified DNA with a solid support bearing a binding partner specific to the binding agent tagged to the amplified DNA, permitting the amplified DNA to bind to the substrate, and removing unincorporated labelled dNTP or unincorporated labelled primer; and

in step (f) determining the amount of label in the amplified DNA and obtaining the amount of virus particles or viral RNA or cDNA in the serum by comparison with a standard curve.

6. An assay according to any one of claims 1 to 4 which comprises:

in step (d) amplifying the cDNA or viral DNA in a nested multi-stage polymerase chain reaction (PCR) using in a first stage a first pair of PCR primers having sequences complementary to portions of the cDNA or viral DNA sequence which define a region of the cDNA or viral DNA such that the portion of the cDNA or viral DNA sequence complementary to the sequence of the transcription primer is outside that region; and in a second stage, a second pair of PCR primers complementary to portions of the cDNA or viral DNA sequence which lie within the region defined by the portions of the cDNA or viral DNA sequence complementary to the first pair of PCR primers; one of the second pair of PCR primers being tagged with a specific binding agent capable of binding to a specific

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binding partner;

in step (e) contacting the amplified DNA with a solid support bearing a binding partner specific to the binding agent tagged to the amplified DNA, permitting the amplified DNA to bind to the substrate, removing unincorporated labelled dNTP and denaturing the amplified DNA to separate the DNA strands;

in step (f) annealing to one of the strands of the amplified DNA a single stranded DNA oligomer probe having a sequence complementary to a target sequence within the DNA fragment, the target sequence having its 5' end at the base immediately adjacent to the base to be analysed to form a probe/target strand duplex,

in step (g1) incubating a first portion of the probe/target strand duplex strand with a DNA polymerase enzyme and labelled deoxynucleotide triphosphate (dNTP) or dideoxynucleotide triphosphate (ddNTP) corresponding to the base to be analysed in a first of the viral forms in the mixture;

in step (g2) incubating a second portion of the probe/target strand duplex strand with a DNA polymerase enzyme and labelled deoxynucleotide triphosphate (dNTP) or dideoxynucleotide triphosphate (ddNTP) corresponding to the base to be analysed in a second of the viral forms in the mixture;

in step (h) removing the unincorporated labelled dNTP or ddNTP; and

in step (i) determining the relative amounts of label bound to the probe in each portion of the probe/target strand duplex, or in each portion of the probe after denaturation and

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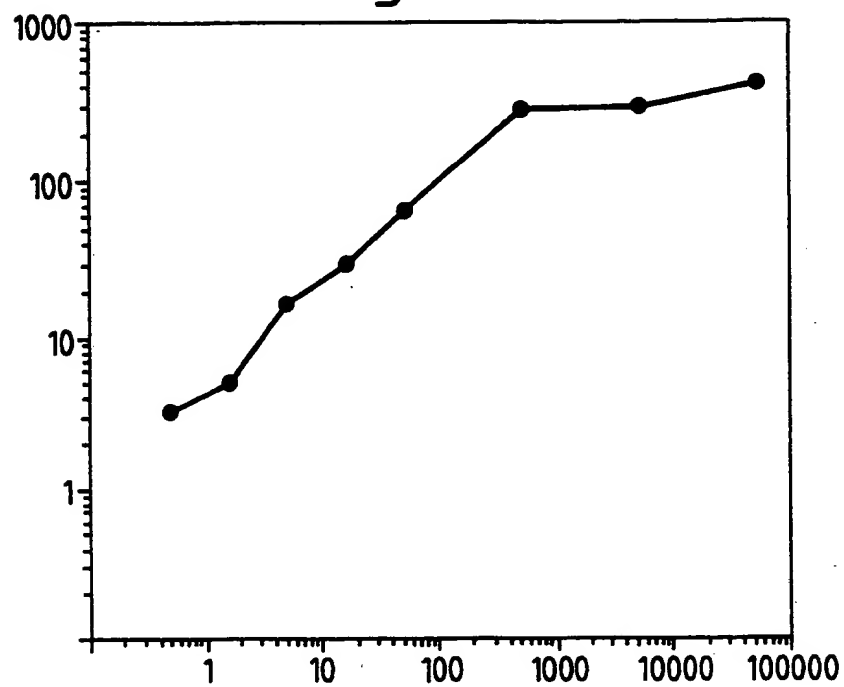
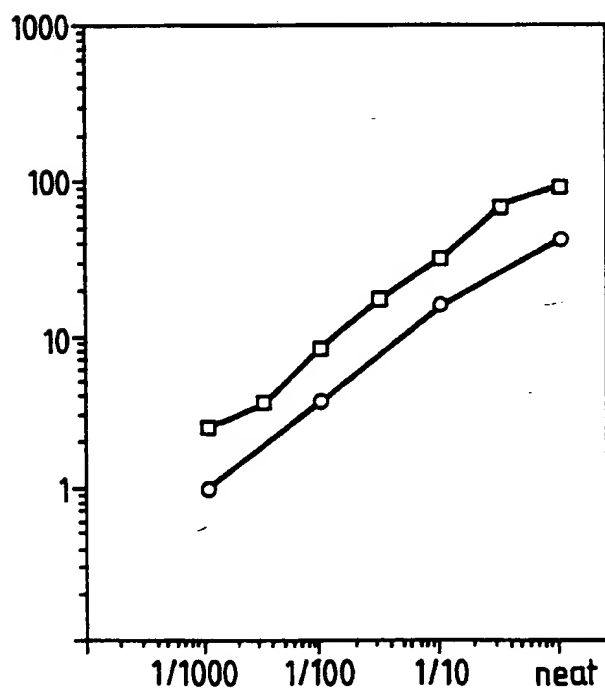
separation from the target strand.

7. Assay according to claim 6 in which in step (f) the base to be analysed is the site of a point mutation and in step (i) the relative amounts are determined of DNA showing different basis at the point mutation site.

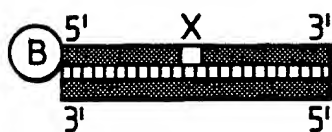
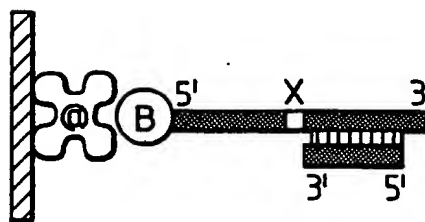
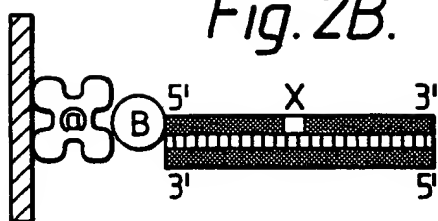
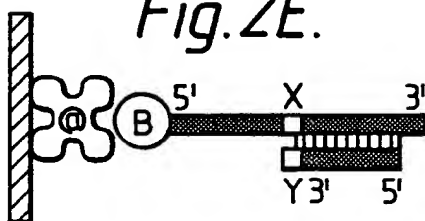
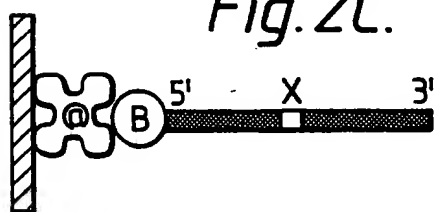
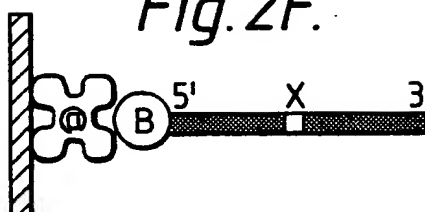
8. Assay according to any one of the preceding claims wherein before the amplification step (d) the sample is divided into two portions, one of which is assayed according to claim 5 and the other of which is assayed according to claim 6 or 7.

9. Use of an assay as claimed in any one of the preceding claims in determining the development of drug resistance in a sample removed from a human or animal patient.

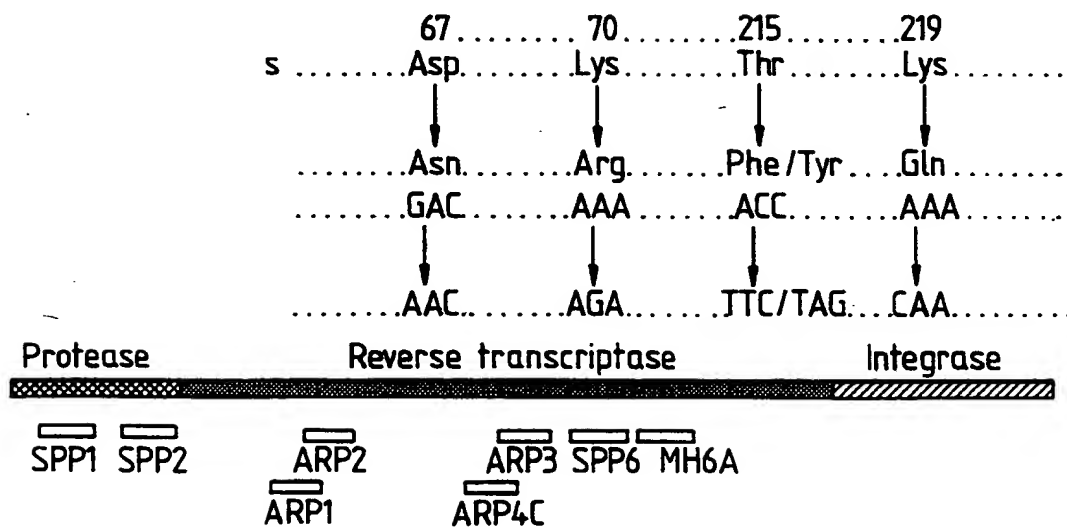
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Fig.1A.*Fig.1B.*

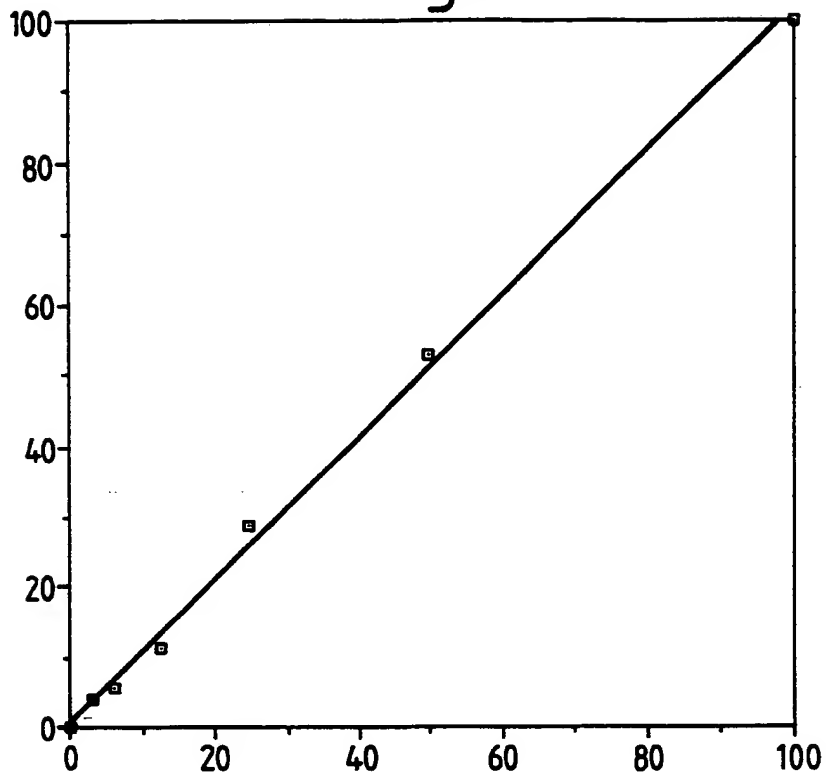
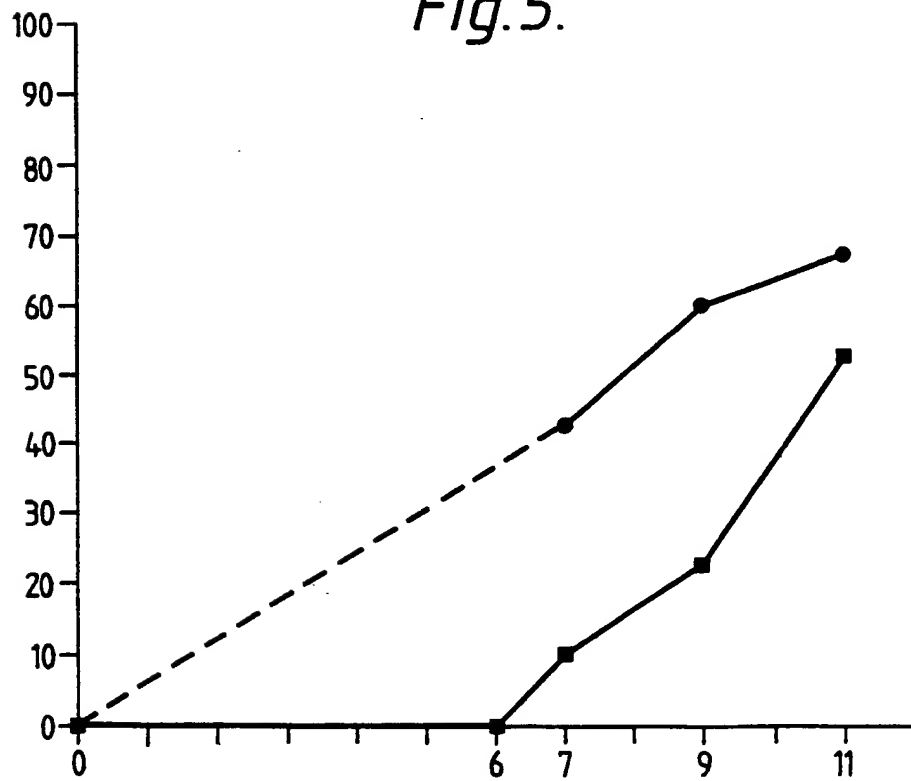
2 / 3

Fig. 2A.*Fig. 2D.**Fig. 2B.**Fig. 2E.**Fig. 2C.**Fig. 2F.*

Y 3' 5'

Fig. 3.

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Fig.4.*Fig.5.***SUBSTITUTE SHEET**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/00745

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12Q1/68; C12Q1/70		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12Q	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ^o	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	EP,A,0 357 011 (ABBOT LABORATORIES) 7 March 1990 see the whole document ---	1-9
Y	EP,A,0 402 997 (AKZO N.V.) 19 December 1990 see the whole document especially example II. ---	1-9
Y	EP,A,0 366 448 (THE GENERAL HOSPITAL CORP.) 2 May 1990 see the whole document ---	1-9
Y	WO,A,9 113 075 (ORION-YHTYMÄ OY) 5 September 1991 see the whole document * ESPECIALLY EXAMPLE 10* ---	1-9
-/--		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>^o Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
22 JULY 1993		0 6. 08. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		OSBORNE H.H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
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Y	EP,A,0 408 918 (CANON KABUSHIKI KAISHA) 23 January 1991 see claims 1-4; example 3	6-9
Y	PROC.NATL.ACAD.SCI. USA vol. 88, February 1991, WASHINGTON pages 1143 - 1147 KUPPUSWAMY 'single nucleotide primer extension to detect genetic diseases' see the whole document	6-9
A	EP,A,0 370 694 (EASTMAN KODAK) 30 May 1990	

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